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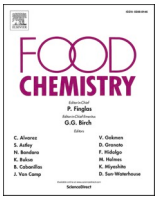

Root-applied glycinebetaine decreases nitrate accumulation and improves quality in hydroponically grown lettuce

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ABSTRACT

Leafy vegetables like lettuce (*Lactuca sativa* L.) naturally have high nitrate content and the European Commission has set maximum level for nitrate in lettuce. Glycinebetaine is an organic osmolyte alleviating plant stress, but its role in leaf nitrate accumulation remains unknown. The uptake of glycinebetaine by lettuce roots, and its potential to regulate lettuce nitrate content and improve plant quality were investigated. Two hydroponic lettuce experiments were conducted with different glycinebetaine application rates (Exp1: 0, 1, 7.5, and 15 mM; Exp2: 0, 1 + 1 + 1, 1 + 10, and 4 mM). Plants were analyzed at varying time points. Root application resulted in glycinebetaine uptake and translocation to the leaves. Glycinebetaine concentrations > 7.5 mM reduced leaf nitrate up to 40% and increased leaf dry matter content. Glycinebetaine showed a positive effect on leaf mineral and amino acid composition. Thus, glycinebetaine could be a novel strategy to reduce the nitrate content in hydroponic lettuce.

1. Introduction

Leafy green vegetables contain plenty of health-promoting compounds. However, consuming leafy vegetables may also increase the intake of nitrate (NO_3^-). An estimated 70–90% of the nitrate in human diets originates from raw leafy vegetables (European Food Safety Authority (EFSA), 2008). The European Commission has classified nitrate as a naturally occurring hazardous substance, and thus the European Commission has set maximum nitrate content levels in spinach (*Spinachia oleracea* L.), fresh lettuce (*Lactuca sativa* L.), and garden rocket (*Eruca vesicaria* subsp. *sativa* (Miller) Thell.) (European Commission Regulation No 1258/2011). However, the nitrate content in lettuce regularly exceeds the set maximum level within the European Union (Colla et al., 2018; European Food Safety Authority (EFSA), 2008). Nitrate accumulates in greenhouse-grown lettuce from the fertilizers. Most plants prefer nitrate over other nitrogen (N) sources, and nitrate is the most commonly used nitrogen source in leafy vegetable production. It does not have growth-restricting effects even at high concentrations, unlike ammonium and urea. After plants have taken up nitrate, it is quickly excluded from the phloem and stored or metabolized further.

Lettuce species are classified as nitrophilic and contain high levels (over 2500 mg kg^{-1} fresh weight) of nitrate (Santamaria, 2006). The level of excessive nitrate uptake and accumulation depends on the growing conditions, lettuce species, and cultivars (Colla et al., 2018). Most important factors affecting the plant nitrate content include light and the nitrate concentration of the fertigation solution. In general, a high nitrate concentration of the fertigation solution (Colla et al., 2018) and low light intensity (Blom-Zandstra & Lampe, 1985) result in high nitrate content of the plant. Lettuce accumulates nitrate as an osmolyte in the dark and under low light intensity, when the availability of photosynthates for organic osmolyte synthesis is limited (Blom-Zandstra & Lampe, 1985). This is due to sucrose being required for the maintenance of both major enzymes of nitrogen metabolism, nitrate reductase, and nitrite reductase (Morcuende et al., 1998).

Osmolytes are usually small organic molecules that plants accumulate within their cells to maintain cellular osmotic pressure equal to that of the external fluid environment. Osmolytes include e.g. sugars, polyols and their derivatives, amino acids and their derivatives (e.g. glycinebetaine (GB) i.e. N, N, *N*-trimethylglycine), methylamines and methylsulfonium solutes, and urea (Yancey, 2005). Glycinebetaine is a non-

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toxic, odorless, and colorless quaternary ammonium compound accumulated by many halophytes and some crop species, although several crop species either synthesize it in physiologically insignificant concentrations or cannot synthesize it at all (Wyn Jones & Storey, 1981). For commercial purposes, GB is extracted mainly from sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll), and seaweeds and is used for example in personal care, cosmetic products, and as a food and feed additive.

Due to its known role as an osmolyte and compatible solute, GB synthesis has been the target of traditional breeding programs and genetic modification (Mäkelä et al., 2019). These attempts are, however, rather time-consuming and costly, and the final outcome is not always predictable. The exogenous application of GB is an alternative, either through the foliage or roots. However, plant tolerance to applied GB concentration varies, and applied GB is diluted as the plant accumulates biomass (Mäkelä et al., 2019).

In commercial lettuce cultivation systems, nitrate accumulation can be limited by growing the plants in a nitrate-free or nitrate-limited solution for a period before harvest, replacing the nitrate with other forms of nitrogen, using nitrate inhibitors, or adjusting the light intensity and its quality and photoperiod (Colla et al., 2018). Illuminating the plants with a mix of red and blue light-emitting diode (LED) light continuously for 48 h before harvest decreases the nitrate content and increases the organic solute content of lettuce (Wanlai et al., 2013). As nitrate is the major form of nitrogen and it plays an important role in lettuce cultivation, decreased nitrate concentrations and other sources of nitrogen may limit yields and deteriorate the quality in intensive and continuous commercial lettuce production (Stagnari et al., 2015). Both changes in light conditions at various growth stages and interruptions of nutrient supplies will cause additional system and labor costs. In addition, various lettuce cultivars and varieties may need different control practices (Reinink, 1993). Therefore, developing a novel management approach is crucial to preventing excess absorption of nitrate without causing remarkable growth suppression or increasing initial and running costs.

As lettuce accumulates nitrate as an osmolyte (Blom-Zandstra & Lampe, 1985) and organic osmolytes affect plant nitrate accumulation (Burns et al., 2011), the hypothesis was that GB applied in fertigation solution in a nutrient film technology (NFT) system is taken up by lettuce plants and results in decreased the nitrate content of lettuce. Furthermore, because nitrate affects the plant nitrogen metabolism, and GB is known to increase plant chlorophyll and protein content (Mäkelä et al., 2019) as well as promote human health (Cholewa et al., 2014), the hypothesis was that added GB can affect the amino acid and mineral nutrient content of lettuce. To our knowledge, this is the first report in which the role of glycinebetaine as regulator of nitrate accumulation in leafy green vegetables has been described.

2. Materials and methods

2.1. Plant material and experimental design

Two experiments were conducted at the Viikki Campus of the University of Helsinki, Finland. In the first experiment, one seed of lettuce (cv. Frillice) was seeded in 0.08-L pots (PR306 ø60 × 51 mm, VEFI EUROPE, Drammen, Norway) containing potting mix (pH 6.0, EC 2.1 mS/cm, VHM 620 R8060 + Fe, Kekkilä Professional, Vantaa, Finland) and grown at a commercial glasshouse company (Oksasen Puutarha, Finland). At 15 days after seeding (DAS), seedlings in the pots were transferred to NFT channels in controlled glasshouse conditions with day/night temperatures and a relative humidity of 20 °C/18 °C and 55–65%, respectively. High-pressure sodium lamps (Masterson-t; Philips Lighting N.V., Eindhoven, The Netherlands) provided a 20-h photoperiod with PPFD of 150 μmol m^{-2} s^{-1} throughout the day at the top of the canopy. Each channel had 34 pots. There were four separate NFT systems, each consisting of four channels split onto four tables in a completely randomized block design with four replicates. The nutrient solution for each NFT system was prepared in 144-L hydroponic containers as a mixture of Vihannes Superex (N—P—K: 9–5-31, Kekkilä Professional, Kekkilä Oy, Finland) and YaraTera Calcinit (N: 15.5, Yara Suomi Oy, Finland). The concentrations of elements in the nutrient solution added were in mM (mg L^{-1}): N 12.85 (180), P 1.39 (43), K 7.83 (306), Ca 4.44 (178), Mg 4.73 (115), S 0.93 (30) and in μM (mg L^{-1}): Fe 16.65 (0.93), Mn 7.83 (0.43), B 24.05 (0.26), Zn 2.14 (0.14), Cu 1.26 (0.08), Mo 0.52 (0.05) and Co 0.17 (0.01). The electrical conductivity (EC) of the solution was set to 1.8 dS m^{-1} and pH to 6. The flow rate of the nutrient solution in the hydroponic system was 12 L h^{-1}. A new nutrient solution was added when 25% of the container's full capacity was used. Nitrate-N concentration, pH and EC were monitored daily. Glycinebetaine (Greenstim, Verdera Oy, Lallemand Plant Care, Finland) was added to the nutrient solution to reach concentrations of 0, 1, 7.5, and 15 mM GB at 29 DAS, when the plant fresh weight (FW) was approximately 59 g. The doses applied were based on several pre-trial tests ranging from 0.01 mM to 35 mM. The GB treatments lasted seven days, during which the target concentration was maintained by adding GB based on the daily analysis results. The containers were washed after each GB treatment, and fresh nutrient solution was added to the containers.

In the second experiment, two lettuce cultivars (Frillice and Exact) were used. The pots were transferred to NFT channels at 17 DAS (cv. Frillice) and at 20 DAS (cv. Exact). Each channel had 16 pots of cv. Frillice and 16 pots of cv. Exact. There were four separate NFT systems, each consisting of four channels split onto four tables in a completely randomized block design with four replicates. Glycinebetaine (Nutristim, Verdera Oy, Lallemand Plant Care, Finland) treatments were begun at two different stages. In the early treatments (Early1), split GB (1 + 1 + 1 mM GB) was added to the nutrient solution three times to reach a 1-mM concentration at 28, 31, and 36 DAS for cv. Frillice and at 31, 34, and 39 DAS for cv. Exact. In the late treatments, GB was added to reach either a concentration of 1 mM (Late1) or 4 mM (Late4) at 31 DAS for cv. Frillice and 34 DAS for cv. Exact. A further 10-mM GB treatment was added to the remaining Late1 plants at 41 DAS for cv. Frillice and 44 DAS for cv. Exact. The control treatment was 0 mM GB. The nutrient solutions were not changed after treatments, but fresh nutrient solution was added to maintain the tank volume.

2.2. Sampling and measurements

In the first experiment, nutrient solutions were sampled and analyzed daily for GB concentration during the treatment. Plant samples were harvested five times: at 24, 29, 36, 41, and 49 DAS. For the first two samplings, at 24 and 29 DAS, one plant of each cultivar was harvested from each channel by cutting the plant from the root collar, totaling four plants per treatment. For the last three samplings, at 36, 41, and 49 DAS, six plants of each cultivar were harvested from each channel, i.e. 24 plants per treatment in total. Harvested plants were cut in half, and the halves were weighed and placed in separate plastic bags. Samples were stored at −20 °C until further analysis. The relative growth rate (RGR), based on fresh weight, was calculated according to Hunt (1982) as $(lnFW_2 - lnFW_1)/(t_2 - t_1)$, in which t is sampling time.

The other half of the frozen plant was freeze-dried (CHRIST Beta 2–8 LD plus, CHRIST Gamma 2–16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 24–96 h under 0.850–0.650 mbar. Dry samples were weighed and ground (Retsch Grindomix GM 200, Retsch GmbH, Germany) into a fine powder. Ground samples were stored at −20 °C until further analysis. Dry matter percentage was calculated as $(DW/FW)x100$, in which DW is sample dry weight.

Plant samples were harvested five times in the second experiment: cv. Frillice at 28, 31, 36, 41, and 45 DAS and cv. Exact at 31, 34, 39, 44, and 49 DAS. For the first sampling, at 28 DAS for cv. Frillice and at 31 DAS for cv. Exact, one plant of each cultivar was harvested from each channel by cutting the plant from the root collar, totaling four plants per

treatment-cultivar combination. For the second sampling, at 31 DAS for cv. Frillice and at 34 DAS for cv. Exact, three plants of each cultivar were harvested from each GB-treated channel. One plant was harvested from each control channel, totaling four plants, and combined to form the control sample. For the third and fourth samplings at 36 and 41 DAS for cv. Frillice and at 39 and 44 DAS for cv. Exact, four plants of each cultivar were harvested from each channel. For the fifth sampling, at 45 DAS for cv. Frillice and at 48 DAS for cv. Exact, two plants of each cultivar were harvested from each Late1 and control channel. All samples were treated similarly as in the first experiment.

2.3. Nitrate analysis

The other half of the sampled and frozen plants was stored for 24 h at 5 °C, followed by at least 2 h at room temperature. The samples were squeezed with a potato press and the liquid was collected in a glass beaker. The nitrate content was analyzed from the liquid with a HORIBA LAQUATwin 741 m for crops (HORIBA Scientific Ltd., Japan). The result was multiplied with a factor of 0.77, according to Näkkilä et al. (2015). In the second experiment, the remaining squeezed liquid from the third and fifth samplings was stored in 50-ml falcon tubes at −20 °C for further nitrate analysis at the Natural Resources Institute, Finland, using flow injection analysis (Lachat Quikchem 8000 FIA analyzer, Zellweger Analytics, USA) and spectrometric detection. In brief, the sample was fed into a continuously flowing buffer solution with an injection valve with constant mixing. A continuously flowing phosphoric acid reagent was admixed and resulted in a red precipitate that was measured spectrophotometrically at an absorbance of 520 nm. The correlation coefficient between the nitrate results obtained with the HORIBA LAQUATwin 741 m and the flow injection analysis was 0.97, and thus the nitrate data we present are based on the HORIBA method.

2.4. Glycinebetaine analysis

Glycinebetaine was extracted from the freeze-dried and ground samples based on the methods of Zhao et al. (2013) and Kojić et al. (2017) with slight modifications. In short, 200 mg of ground freeze-dried sample was weighed into 30-ml centrifuge tubes and 10 ml of methanol was added. Samples were shaken for 10 min at 22 °C in a water bath, followed by ultrasonication for 30 min at 22 °C (Branson 5510, Emerson Co., USA), after which the extract was centrifuged at 8000 rpm for 10 min. The supernatant was paper-filtered (Qualitative paper 415, pore size 12–15 μm, VWR, Leuven, Belgium). The pellet was suspended in 5 ml of methanol, and the centrifugation and filtering were repeated. Methanol was evaporated at 45 °C under the stream of nitrogen (Pierce Reacti Vap III + Pierce Reacti-Therm III, Thermo Scientific Inc.). The residue was reconstituted in 400 μl of acetonitrile:water mixture (9:1 v/v) and syringe-filtered (0.2 μm, PALL, Cornwall, UK) in a UHPLC vial. Samples were stored at −20 °C until GB analysis. Samples of nutrient solutions were diluted in acetonitrile, at a ratio of either 1:5 or 1:1 (v/v), and syringe-filtered as described above.

Glycinebetaine content was determined from the nutrient solution samples and extracted plant samples with a Waters Acquity ultra-high-performance liquid chromatography (UHPLC) system (Milford, MA) equipped with a light-scattering detector using a Cortecs HILIC column (2.1 × 150 mm, 2.7 μm; Waters). The autosampler was set at room temperature and the column at 30 °C. The separation of GB was achieved by isocratic elution with the mixture of acetonitrile and 30 mM ammonium acetate (8:2, v/v) at a flow rate of 0.4 ml min^{-1}. The detector settings were: drift tube temperature was at 45 °C, nebulizer was set to heating at 45% of the power level (27 °C), gas flow rate was 2.92 L min^{-1}, and the pressure was 50.0 psi. Injection volumes were 3–10 μl and elution time for GB was 4.5–6.0 min. Each analysis run included a six-point standard curve based on known concentrations (100–1000 μg ml^{-1}) of GB (Betaine monohydrate ≥ 99%, Sigma-Aldrich Chemie, Steinheim, Saksa). Chromatographic data were collected and processed using the Waters Empower 2 software (version 2, Hitachi High Technologies Inc., CA, USA).

2.5. Mineral analysis

Trace elements (B, Cu, Fe, Ca, K, Mg, Na, P, and S) were analyzed from the freeze-dried and ground plant samples of the fourth samplings of the first and second experiments. For extraction, 250-mg samples were weighed into polytetrafluoroethylene (PTFE) Teflon tubes (CEM, Matthews, North Carolina, USA) and 6 ml of 15.2 M nitric acid (67–69% w/v, VWR International BVBA, Leuven, Belgium) and 1 ml of 9.8 M hydrogen peroxide (30% w/v, Merck KGaA, Darmstadt, Germany) were added for microwave digestion (MARSXpress, MARS 240/50, CEM, Matthews, NC, USA). Digested samples were filtered through paper (Whatman, Grade No. 42, pore size 2.5 μm, GE Healthcare, UK), diluted in purified water, and stored at −20 °C. Elemental analysis was conducted with inductively coupled plasma-optical emission spectrometry (ICP-OES) (iCAP 6200, Thermo Fisher Scientific, Cambridge, UK) with every 20th sample as standard. The total N content was analyzed from the freeze-dried and ground plant samples (200 mg) with the Dumas combustion method using Vario MAX CN (Elementar Analysensysteme GmbH, Hanau, Germany).

2.6. Amino acid analysis

Freeze-dried and ground plant samples were used for amino acid analyses. Amino acids, excluding tryptophan, were isolated according to the European Union directive (European Commission, 1998). Basic hydrolysis was used for isolating the tryptophan. In short, 200 mg of ground sample was weighed in a glass tube and 10 ml of 4 M NaOH was added. Samples were incubated in an oven at 110 °C for 24 h, after which the samples were diluted to 50 ml and neutralized by 0.32 M HCl.

Free amino acids were extracted by homogenization of a 30-mg ground sample in 50%/50% v/v methanol/mQ water 3 ml with TissueRuptor (Qiagen) and centrifuged at 13,000 g for 10 min (Collado-Gonzalez et al., 2014). After filtration, 20 μl of sample was transferred in a 1.5-ml vial and 60 μl of borate buffer was added, and the mixture was shaken for 5 s. An aliquot of 20 μl of 6-aminoquinolyl-*N*-hydroxycuccinimidyl carbamate in acetonitrile (3 mg/ml) was added and the mixture was shaken for 5 s. Before incubation at 55 °C for 10 min, samples were allowed to stand at room temperature for one minute.

Amino acids were analyzed with an ACQUITY UPLC system (Waters Milford, MA, USA) consisting of an Acquity photodiode array (PDA) optical detection system. A Waters BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters Milford, MA, USA) was used. The flow rate was 0.7 ml/min and the column temperature was 55 °C. The injection volume was 1 μl and the detection wavelength was 260 nm. The solvent system consisted of two eluents: (A) AccQ·Tag ultra eluent A concentrate (5%, v/v) (10%v/v with tryptophan) and water (95%, v/v); (B) AccQ·Tag ultra eluent B. The following gradient elution was used: 0–0.54 min, 99.9% A–0.1% B; 5.74 min, 90.9% A–9.1% B; 7.74 min, 78.8% A–21.2% B; 8.04 min, 40.4% A–59.6% B; 8.70–10 min, 99.9% A–0.1% B. Empower 2 (Waters Milford, MA, USA) software was used for system control and data acquisition.

2.7. Statistics

The leaf number, fresh weight, dry matter, nitrate, mineral, and GB contents of the lettuce samples collected at different time points were subjected to two-way analysis of variance to reveal the effects of GB treatments, lettuce species, and harvesting dates as fixed effects. Pairwise comparisons were made using Tukey's multiple range test, and significant differences between treatment means were considered when the *p*-values were < 0.05. Analyses were performed with R software (Version 4.0.2; R Development Core Team, Vienna, Austria).

3. Results

3.1. Plant growth

In both experiments, the effect of the root-applied GB on lettuce biomass accumulation depended on the used concentration (Tables 1 and A1). Shoot fresh weight was not affected by GB concentration ranging from 1 to 4 mM when compared with the control (0 mM GB). However, when GB concentration was ≥ 7.5 mM, shoot fresh weight accumulation immediately decreased compared with the control (Tables 1, A1 and A2). The observed growth effect was independent of lettuce size and variety. The decrease in biomass accumulation of lettuce following the two highest GB concentrations (7.5 and 15 mM) in the first experiment was reversible, as the growth recovered to 42 DAS. At that time, the slope of the growth curve and the relative growth rate were close to those of the control (data not shown). Assuming that the lettuce fresh weight is 150 g plant^{-1} in commercial cultivation at harvest, the two highest GB concentrations would have delayed the harvest by approximately six days. With the lower GB concentrations used in the second experiment, no such delay would have occurred (Fig. A1). In addition, it is important to note that the GB application did not affect leaf number (plastochron) in any harvest when compared with the control (Tables 1 and A1) and thus the observed shoot fresh weight reductions were due to the decreased leaf weight. Moreover, the visual observations indicated that leaf area per plant decreased due to the increasing GB concentration.

Table 1
Plant leaf number, plant fresh weight (FW), dry mass (DM), and glycinebetaine (GB) content in lettuce cv. Frillice in response to GB concentration in the nutrient solution in the first experiment at various harvest times.

Harvest (DAS)	GB mM	Leaf number	Plant FW (g)	Plant DM (%)	GB, μg/g (FM)
1 (24)	0 mM	9^{a}	21.9^{a}	nd	nd
	1 mM	8^{a}	21.5^{a}	nd	nd
	7.5 mM	8^{a}	21.1^{a}	nd	nd
	15 mM	9^{a}	24.1^{a}	nd	nd
	S.E.M	0.3	1.01	nd	nd
	p-value	0.497	0.228	nd	nd
2 (29)	0 mM	17^{a}	62.5^{a}	7.0^{a}	nd
	1 mM	18^{a}	60.8^{a}	7.3^{a}	nd
	7.5 mM	18^{a}	56.2^{a}	6.6^{a}	nd
	15 mM	18^{a}	55.7^{a}	6.8^{a}	nd
	S.E.M	0.3	3.67	0.2	nd
	p-value	0.302	0.507	0.152	nd
3(36)	0 mM	26^{a}	147.6^{b}	5.3^{a}	0.0^{a}
	1 mM	26^{a}	141.9^{b}	6.0^{a}	2.3^{b}
	7.5 mM	27^{a}	94.4^{a}	7.2^{b}	5.4^{c}
	15 mM	27^{a}	84.6^{a}	8.0^{b}	8.5^{d}
	S.E.M	0.5	2.93	0.22	0.32
	p-value	0.394	<0.001	<0.001	<0.001
4(41)	0 mM	37^{a}	272.1^{d}	4.9^{a}	0.0^{a}
	1 mM	38^{a}	249.1^{c}	5.1^{a}	2.0^{b}
	7.5 mM	38^{a}	160.9^{b}	6.1^{b}	4.6^{c}
	15 mM	37^{a}	129.3^{a}	7.3^{c}	9.1^{d}
	S.E.M	0.5	3.78	0.125	0.40
	p-value	0.337	<0.001	<0.001	<0.001
4(49)	0 mM	nd	457.0^{b}	5.2^{a}	0.0^{a}
	1 mM	nd	482.5^{b}	5.0^{a}	0.8^{a}
	7.5 mM	nd	308.0^{a}	5.5^{a}	2.0^{b}
	15 mM	nd	283.5^{a}	5.7^{a}	4.0^{c}
	S.E.M	nd	10.26	0.21	0.237
	p-value	nd	<0.001	0.113	<0.001

DAS = Days after seeding; S.E.M. = Standard error of the mean; nd = not determined. Significance tested at $P < 0.05$ Tukey test. Significant differences ($p < 0.05$) between means are indicated by different letters.

3.2. Glycinebetaine in the nutrition solution

In both experiments, the GB concentration of the nutrient solution was analyzed daily. In the first experiment, GB concentration began decreasing gradually within two days and was approximately 80% of the original concentration in all treatments (data not shown). Thereafter, additional GB was supplemented every other day into the nutrient solution to maintain the intended GB concentration. The total amount of GB (molecular weight 117.15 g mol^{-1}) added into each fertigation tank (144 L) to keep the original concentration (1, 7.5 and 15 mM) during the treatment period was as follows: 54.5, 340.5 and 544.7 g, respectively. No supplementary GB was added in the second experiment, except in the split application treatments (Early1; GB 1 + 1 + 1 mM and Late1; GB 1 + 10 mM). The decrease of GB in the 4-mM solution was linear (R^2 = 0.869) over time in the second experiment.

3.3. Glycinebetaine and nitrate accumulation

The nitrate content of lettuce leaves decreased following GB application and the reduction was dose-dependent in both experiments (Fig. 1 and A1). The effect of GB on the decrease of leaf nitrate content was reversible over time, and the reversibility depended on the GB concentration in the nutrient solution. The decrease in nitrate content was prolonged for 15 days in plants that received the highest GB concentration (15 mM), but the decrease was prolonged for slightly over 5 days in plants that received the lowest (1 mM) GB concentration. Reversibility did not depend on the lettuce cultivar. It also important to note that the nitrate content of the lettuce leaves also decreased following the very late GB application (10 mM) for large lettuce plants compared with the control in the 5th sampling of the second experiment.

Furthermore, the root application of GB showed a positive effect on increasing the GB content in lettuce leaves. The increase occurred in a concentration-dependent manner in the first experiment (Table 1), and the trend was similar in the second experiment (Table A1). A clear peak was observed in leaf GB content, which declined gradually over time. The plant total uptake of GB (μg GB plant^{-1}) reached its maximum value at 41 DAS in each treatment. No GB was found in the control plants.

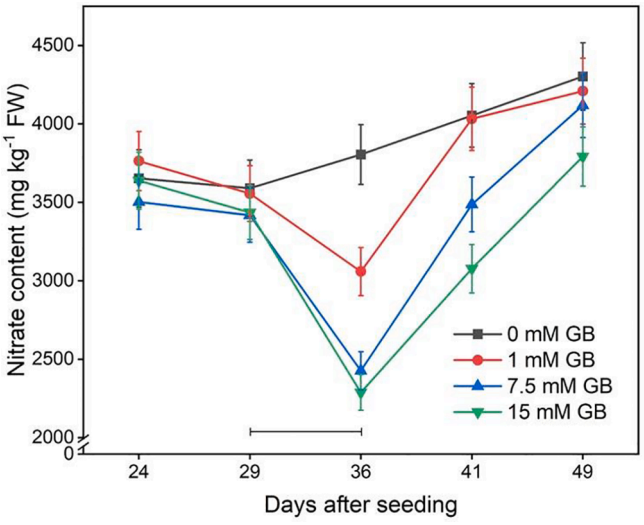

Fig. 1. Effect of glycinebetaine (GB) concentration on nitrate accumulation in lettuce cv. Frillice in the first experiment. The application period is marked by a horizontal arrow. FW = Fresh weight.

In the first experiment, we observed a strong negative interaction between nitrate accumulation in the leaves and the leaf GB content (Fig. 2). An indistinguishable negative interaction was also found in the second experiment (Fig. 2). To decrease the nitrate accumulation from 30% to 40%, the data in Fig. 2 predict that the GB content in the lettuce leaves should be over 5 μg g^{-1} FW (90 μg g^{-1} DW).

3.4. Leaf dry matter content and leaf senescence

The leaf dry matter content, determined for the entire growing period in the first experiment, was increased by the root-applied GB concentration in a concentration-dependent manner (Table 1). In addition, the two highest GB concentrations (7.5 and 15 mM) led to increases in leaf dry matter content instantly after GB application, after which the leaf dry matter content decreased. Leaf dry matter content in plants cultivated with the lowest GB concentration (1 mM) was comparable with that of the control (GB 0 mM), which decreased over the whole growing period. Compared with the control (GB 0 mM), the highest GB concentration (4 mM) in the second experiment resulted in increases in the leaf dry matter content of both lettuce cultivars (Tables A1 and A2). Moreover, leaf senescence determined as the proportion of etiolated leaves in the lettuce cv. Exact at 44 DAS was decreased by the root-applied GB in a dose-depended manner (Fig. A2). It is notable that leaf senescence was virtually absent in the leaves of plants treated with triple (1 + 1 + 1 mM) or single (4 mM) GB application (Fig. 3).

3.5. Leaf mineral and amino acid composition

The lettuce cv. Frillice leaf mineral composition was analyzed once during the first experiment at 41 DAS. The sampling time was chosen based on size and cultivation period, which reflect the common practice in commercial lettuce production. All mineral contents except potassium increased in the edible lettuce leaves in a concentration-dependent manner due to the root-applied GB (Table 2). Similar trends were observed in the second experiment with the lettuce cv. Frillice (Table A3) but not with cv. Exact (Table A4). Generally, the leaf total nitrogen content increased and leaf nitrate content decreased as the GB concentration in the nutrient solution increased. The lowest proportion (56%) of nitrogenous compounds other than nitrate was in the control leaves (GB 0 mM), whereas the highest (75%) was in the GB 15 mM treatment. Although the content of GB nitrogen in the leaves increased due to GB application, its proportion in nitrogenous compounds was negligible.

The lettuce leaf free and hydrolyzed amino acid compositions were analyzed in the first experiment at 41 DAS. The analysis of free amino acids showed that GB application resulted in higher histidine, glutamine, arginine, gamma butyric acid, and proline contents compared with untreated lettuce (Table 3). Glycinebetaine treatment affected proline most remarkably, as its content increased 5-fold with GB 15 mM compared with the control. Compared with untreated lettuce, lower contents of asparagine, glutamic acid, and alanine were observed due to GB application. The results of the hydrolyzed amino acid analysis revealed that GB application increased the content of nearly all the amino acids compared with the control (Table A5). From a human nutritional viewpoint, it is important to note that GB application and the hydrolysis of leaf dry matter increased the content of all the nine essential amino acids (histidine, tryptophan, lysine, valine, isoleucine, leusine, phenylalanine, tryptophan, and methionine) in lettuce leaves. Pearson correlation coefficients confirmed that the statistically significant changes in the abovementioned free and hydrolyzed amino acids depended on the GB application concentration (Tables A6 and A7).

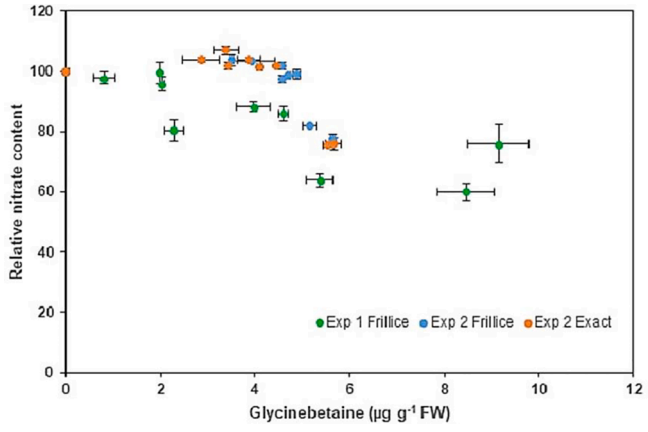

Fig. 2. Interaction between nitrate accumulation in the lettuce leaves and the leaf glycinebetaine (GB) content. FW = Fresh weight; Exp = Experiment.

4. Discussion

4.1. Root-applied glycinebetaine improved lettuce growth and quality

Our results indicated that lettuce does not naturally accumulate GB, which agrees with earlier report by Shams et al. (2016). However, the root-applied GB was taken up and accumulated in the lettuce leaves, with a clear trend of increased GB content in the leaves following an increased GB application rate in the NFT cultivation system. Root application of GB has been studied earlier in other crops, such as in rice (*Oryza sativa* L.) (Shahbaz & Zia, 2011), oilseed rape (*Brassica napus* L. ssp. *oleifera* (Metzg.) (Athar et al., 2015), and tomato (*Lycopersicon esculentum* L.) (Heuer, 2003), and have been found to improve the performance of the plants under various stress conditions, e.g. salinity and water deficit.

A high application rate (>10 mM) of GB seemed to limit phytomass accumulation due to the decreased leaf weight. This may indicate limited nitrate availability in the active cell pool (cytosol or/and vacuole) despite the light intensity and nitrate concentration in the NFT solution being optimized for lettuce growth. In addition to the limited nitrate availability in plant cells, GB may also limit the formation of temporary carbohydrate storages accumulating during the day. Assimilated carbohydrates are allocated in the vacuoles and chloroplasts during the day and partitioned into structural components and biomass during the night (Mitchell et al., 1992). In the presence of GB, the carbohydrate demand as osmolytes may decrease, triggering a feedback loop restricting photosynthesis, resulting in decreased leaf weight and leaf area. Decreased leaf area will diminish the light capture capacity of an individual lettuce plant. In a practical NFT cultivation system, the minor decrease in biomass accumulation with higher application rates (>10 mM) can be leveled off by extending the cultivation time for a few days.

Glycinebetaine markedly reduced the senescence of old lettuce leaves normally senescing first in mature plants. Glycinebetaine is known to alleviate various and specific plant stress responses (Mäkelä et al., 2019). However, it seems that GB delayed the leaf senescence process even under optimal conditions with adequate light, nutrients, and water availability. Glycinebetaine can be hypothesized to delay leaf senescence by affecting the programmed cell death (apoptosis). In animal cells, GB prevents the release of cell death mediators from the mitochondria and inhibits mitochondria-mediated cell death and apoptosis (Ommati et al., 2020).

According to Lim et al. (2007), leaf senescence is an integral part of leaf development, involving various physiological processes such as chloroplast degradation. As chloroplasts degrade, photosynthesis is compensated by the catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA (Tamary et al., 2019). Finally, the senescence results in leaf yellowing and nutrient loss in lettuce crops, limiting yield formation and increasing postharvest spoilage (Gregersen et al., 2013). Retaining the physiological activity of the oldest lettuce leaves with a moderate GB concentration (1–4 mM) results in improved

Fig. 3. Appearance of lettuce plants (cv. Exact) 44 days after seeding in the second experiment. The plants were cultivated with different concentrations of glycinebetaine (GB) in the nutrient solution. From left to right: Late4 = 4 mM GB, Early1 = 1 + 1 + 1 mM GB, and Control = 0 mM GB added in the nutrient solution.

Table 2
Mineral contents in leaves of lettuce cv. Frillice in response to glycinebetaine (GB) concentration in the nutrient solution in the first experiment at 41 days after sowing.

GB Treatment	C	N	K	Ca	Mg	P	S	Fe	Na	B	Cu
	(g/kg FW)			(mg/kg FW)							
0 mM	17.86^{a}	2.03^{a}	4.20^{b}	361.0^{a}	110.4^{a}	278.5^{a}	120.0^{a}	2.0^{a}	26.1^{a}	1.6^{a}	0.2^{a}
1 mM	18.04^{a}	2.22^{ab}	3.85^{ab}	402.2^{ab}	116.2^{a}	294.3^{a}	122.3^{a}	2.5^{b}	27.3^{a}	1.7^{ab}	0.3^{b}
7.5 mM	23.16^{b}	2.47^{bc}	3.45^{a}	362.7^{a}	132.7^{a}	314.9^{ab}	125.5^{ab}	3.8^{c}	28.9^{ab}	1.9^{b}	0.3^{b}
15 mM	28.34^{c}	2.75^{c}	3.78^{a}	428.3^{b}	167.3^{b}	344.6^{b}	137.6^{b}	4.2^{c}	32.2^{b}	2.2^{c}	0.3^{b}
S.E.M (df = 3)	0.723	0.064	0.092	10.18	6.49	8.33	2.86	0.12	0.85	0.06	0.01
P-value (<0.05)	<0.001	<0.001	0.002	0.003	0.001	0.002	0.008	<0.001	0.003	<0.001	0.014

FW = Fresh weight; S.E.M. = Standard error of the mean; Significance tested at $P < 0.05$ Tukey test. C = carbon: N = nitrogen; P = phosphorus; K = potassium; S = sulphur; Ca = calcium; Mg = magnesium; Fe = iron; B = boron; Cu = copper. Significance tested at $P < 0.05$ Tukey test. Significant differences ($p < 0.05$) between means are indicated by different letters.

performance of the whole crop.

Lettuce and other leafy vegetables are used in salad mixtures, and their production and consumption are increasing continuously worldwide. Thus, lettuce will contribute progressively to the nutritional content of diets, as reviewed by Kim et al. (2016). Our results indicate that the diminished senescence of the outer lettuce leaves, higher mineral content, and increased content of the hydrolyzed amino acids, including essential amino acids, particularly through the GB application, improve the nutritional value of lettuce products consumed as fresh salads. In addition, Baslam et al. (2013) observed that the outer leaves of widely grown lettuce cultivars contain higher levels of potentially health-promoting compounds, such as anthocyanins and carotenoids, than the inner leaves, and stripping off the outer leaves decrease their contents.

In addition to the improved nutritional composition and product value, GB application has significant economic impact in commercial lettuce cultivation and the product value chain. During harvest, the outer and senesced leaves are usually removed by hand to improve product acceptance and value. Among tissue decay issues, product color alone is a very important parameter that influences consumer preferences (Pace et al., 2014). The elimination of external leaves with lowered greenness intensity due to the degradation of chlorophyll pigments increases the labor costs of production.

Moreover, GB application resulted in increased dry matter content in lettuce leaves. High dry matter content correlates with improved visual quality and its retention during the post-harvest life of leafy vegetables (Tudela et al., 2017). Recently Min et al. (2021) observed a good correlation between the lettuce shelf life and the dry matter percentage at harvest, the latter being directly related to the improved levels of carbohydrates. In addition to GB presence, the increased free proline and gamma butyric acid content observed in the lettuce leaves due to GB application suggests improved quality preservation. An increasing number of studies have demonstrated that exogenous GB treatment has beneficial effects on quality maintenance in postharvest fruits and vegetables (Sun et al., 2020). Among other studies, they showed that exogenous GB treatment significantly increased antioxidant enzyme activities and gene expression, including superoxide dismutase, catalase, and ascorbate peroxidase, but also decreased the activity and transcript levels of lipoxygenase, resulting in enhanced protection of harvested and fresh plant products from oxidative damage. In addition, proline plays a significant role in protein protection and reactive oxygen species (ROS) scavenging to protect the cell against oxidative damage, resulting in stabilization of the membrane and subcellular structures. Thus, the accumulation of osmolytes, such as GB and proline, in lettuce leaves is expected to balance the cytoplasmic osmotic potential and scavenges ROS to resist abiotic stress over the logistics network of fresh lettuce products to the end customer.

4.2. *Glycinebetaine application decreased nitrate accumulation*

Root-applied GB decreased the nitrate accumulation in the lettuce leaves of the tested NFT cultivation system. The magnitude of the nitrate decrease depended on the GB concentration in the nutrient solution. This finding has significant commercial value, as the nitrate accumulation in the greenhouse and plant factory cultivated leafy vegetables is strictly regulated by the EU (European Food Safety Authority (EFSA), 2008). Nitrate is known to have potentially harmful effects on consumers when ingested, as it is readily converted into nitrite in human saliva and in the gastrointestinal tract (Pannala et al., 2003). Nitrite is toxic, as it has the ability to combine with haemoglobin to form methaemoglobin, which impairs the delivery of oxygen to human tissues (Mensinga et al., 2003).

In recent decades, nitrate applied as a nitrogen source has been realized to frequently accumulate in leafy vegetables, yet controlling its accumulation has proved challenging (Santamaria et al., 2001). Various nutritional approaches have been suggested to reduce nitrate accumulation in lettuce, including ending the crop N supply some few days before harvesting (Santamaria et al., 2001), replacing nitrate N with

Table 3
Free amino acid content of lettuce cv. Frillice in response to glycinebetaine (GB) concentration in the nutrient solution in the first experiment at 41 days after sowing.

GB Treatment	His	Asn	Ser	Gln	Arg	Gly	Asp	Glu	Thr	Ala	GABA	Pro	Lys	Tyr	Met	Val	Ile	Leu	Phe	Trp
	(g/kg DM)																			
0 mM	0.19^{a}	2.41^{a}	1.10^{a}	19.89^{a}	0.78^{a}	0.22^{a}	0.65^{b}	2.38^{b}	0.83^{a}	0.84^{b}	1.38^{ab}	0.24^{a}	0.13^{a}	0.30^{a}	0.02^{a}	0.51^{a}	0.27^{a}	0.21^{a}	0.19^{a}	0.06^{a}
1 mM	0.19^{a}	3.32^{a}	1.10^{a}	21.08^{a}	0.86^{a}	0.20^{a}	0.63^{b}	2.21^{b}	0.81^{a}	0.84^{b}	1.23^{a}	0.29^{a}	0.13^{a}	0.32^{a}	0.02^{a}	0.52^{a}	0.27^{a}	0.21^{a}	0.19^{a}	0.06^{a}
7.5 mM	0.22^{a}	3.25^{a}	0.97^{a}	26.69^{b}	1.29^{b}	0.21^{a}	0.63^{b}	2.14^{ab}	0.70^{a}	0.69^{a}	1.35^{ab}	0.60^{b}	0.13^{a}	0.30^{a}	0.02^{a}	0.43^{a}	0.23^{a}	0.18^{a}	0.17^{a}	0.06^{a}
15 mM	0.28^{b}	2.95^{a}	0.93^{a}	28.01^{b}	1.60^{c}	0.22^{a}	0.53^{a}	1.89^{a}	0.68^{a}	0.61^{a}	1.59^{b}	1.21^{c}	0.14^{a}	0.29^{a}	0.01^{a}	0.43^{a}	0.24^{a}	0.18^{a}	0.15^{a}	0.07^{a}
S.E.M	0.016	0.323	0.087	1.734	0.085	0.011	0.026	0.091	0.067	0.081	0.085	0.084	0.027	0.038	0.003	0.053	0.033	0.027	0.018	0.012
p-value (<0.05)	<0.001	0.057	0.169	0.001	<0.001	0.502	0.004	0.001	0.102	0.034	0.009	<0.001	0.969	0.818	0.198	0.249	0.5	0.523	0.14	0.745

DM (dry mass); S.E.M. = Standard error of the mean; His = Histidine; Asn = Asparagine; Ser = Serine; Gln = Glutamine; Arg = Arginine; Gly = Glycine; Asp = Aspartic acid; Glu = Glutamic acid; Thr = Threonine; Ala = Alanine; GABA = Gamma aminobutyric acid; Pro = Proline; Lys = Lysine; Tyr = Tyrosine; Met = Methionine; Val = Valine; Ile = Isoleucine; Leu = Leucine; Phe = Phenylalanine; Trp = Tryptophan. Significance tested at P < 0.05 Tukey test. Significant differences ($p < 0.05$) between means are indicated by different letters.

chloride or sulphate a few days prior to harvesting (Inal & Tarakcioglu, 2001), and increasing the potassium concentration in the nutrient solution (Ruiz & Romero, 2002). However, these methods have been utilized with varying success. For instance, Liu & Shelp (1996) found that the total plant N and nitrate content did not decrease upon chloride treatment. Inhibition of nitrate uptake due to chloride or sulphate application largely depends on the plant species and on the concentrations of chloride or sulphate with the nitrate in the nutrient solution. Some earlier studies have also shown potassium applications to have little effect in reducing nitrate accumulation in plants (Drlik & Rogl, 1992). In contrast, potassium application may reportedly increase the uptake and transport of nitrates to the shoots, thus promoting the metabolism and utilization of nitrate, resulting in reduced nitrate accumulation in the leaves (Ruiz & Romero, 2002).

Our results indicate that root application of GB may provide an efficient and clear-cut method for controlling nitrate accumulation in commercial lettuce cultivation. In addition, our results indicate that a simplified hydroponic solution constituted with a single organic osmolyte can significantly decrease the nitrate content of hydroponic lettuce after short-term cultivation. Glycinebetaine in the NFT solution can limit nitrate accumulation in lettuce leaves through three hypothetical mechanisms. These alternatives do not necessarily exclude each other but may instead have a simultaneous effect, and even complement each other. In the first alternative, GB in the NFT solution disturbs nitrate uptake. Plants preferably utilize nitrate over other nitrogen forms, and the root uptake rate of nitrogen complies with Michaelis–Menten kinetics. Song et al. (2016) have shown that Chinese kale (*Brassica oleracea* L. var. *alboglabra* (L.H. Bailey) Musil) utilized inorganic N preferentially over organic nitrogen, but its nitrate uptake rate was expressively reduced by adding other forms of nitrogen, such as ammonium, urea, and glycine, into the nutrient solution. However, the precise mechanism of decreased nitrate uptake by other nitrogen forms remains unknown. Although GB is not considered a plant nutrient, it seems that its presence in the nutrient solution can reduce nitrate uptake.

The second alternative assumes that both GB and nitrate have an osmoregulatory role in lettuce. The known role of GB as an osmoticum in plant cells is believed to be related to its effects in improving enzyme and membrane integrity, along with mediating the osmotic adjustment in plants experiencing stress (Mäkelä et al., 2019). The superfluous accumulation of GB occurs predominantly under low light conditions. In addition, certain plant genotypes are prone to nitrate accumulation even under ample light conditions with an overdose of nitrate fertilization. Under these conditions, nitrate acts as an osmoticum (Burns et al., 2011), as it is used to counterbalance any decrease in soluble organic compounds, such as sugars and organic acids, which typically adjust the major part of the osmotic balance in the plant cells. Thus, under limited photosynthesis, nitrate maintains the osmotic potential and generates the turgor needed for leaf expansion. Burns et al. (2011) also proposed a sophisticated isoosmotic mechanism model showing that a 1:1 exchange occurs between nitrate concentrations and the sum of all other endogenous osmotica throughout growth. As a negative correlation was observed between nitrate and GB content in the lettuce leaf tissue, the applied GB possibly replaced nitrate as the osmoticum, resulting in the decrease of nitrate. Furthermore, once the GB content decreased in the lettuce tissue due to the lettuce phytomass increase, the nitrate content increased again. This indicates that the nitrate accumulation response to GB was reversible and GB was once again potentially replaced by nitrate as an osmoticum.

The third mechanism is based on a recent study by Ota et al. (2020), who proposed that plants integrate shoot N status and root N status in the leaves and systemically regulate the efficiency of root N acquisition with a phloem-mobile CEPD-like 2 (CEPDL2) polypeptide. The loss of CEPDL2 in mutant plants led to a reduction in shoot nitrate content and plant biomass. Mutant plants also exhibited decreased expression of the nitrogen transporter genes NRT2.1 and NRT1.5, of which NRT2.1 is involved in high-affinity nitrate uptake and NRT1.5 loads nitrate into

the xylem for root-to-shoot translocation. As our GB responses involved reduced biomass accumulation with reduced nitrate content in lettuce leaves, we suggest that GB may play a yet uncharacterized role in CEPDL2 regulation.

5. Conclusions

Root-applied GB decreased the nitrate content in the leaf tissues of lettuce. Decreased leaf nitrate content was more manifest with high (>7.5 mM) GB applications, which reflects improved quality. Increasing the GB application rate reduced the shoot fresh weight and increased the mineral and hydrolysed amino acid compositions of the lettuce leaves. High nitrate accumulation rates may probably trigger increased GB uptake and accumulation, as observed with the lettuce cultivars. In addition, results show that the tested system for hydroponic solution may be feasible, functional, and practical for soilless cultivation to control nitrate content in leafy vegetables before harvest. The administration of protective and antioxidant agents, such as GB, may be of qualitative value in lettuce production to prevent their leaf senescence and loss of valuable produce. This may also decrease food loss under intensive plant production with high input of cultivation resources. Additional studies are required to investigate the role of root-applied GB as an osmoticum, the regulation of the source–sink relationship, senescence, along with product quality maintenance over the logistics network from the farm to the end consumers.

CRediT authorship contribution statement

Kari Jokinen: Conceptualization, Project administration, Funding acquisition, Methodology, Supervision, Investigation, Validation, Formal analysis, Visualization, Resources, Writing - original draft, Writing - review & editing. **Anna-Kaisa Salovaara:** Investigation, Validation, Visualization, Resources, Writing - original draft, Writing - review & editing. **Daniel O. Wasonga:** Formal analysis, Visualization, Resources, Writing - original draft, Writing - review & editing. **Minnamari Edelmann:** Methodology, Supervision, Investigation, Validation, Visualization, Resources, Writing - original draft, Writing - review & editing. **Ilkka Simpura:** Investigation, Methodology Validation, Visualization, Resources, Writing - original draft, Writing - review & editing. **Pirjo S.A. Mäkelä:** Conceptualization, Project administration, Funding acquisition, Methodology, Supervision, Investigation, Validation, Visualization, Resources Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jokinen Kari and Mäkelä Pirjo have patent #US20200352115A1 pending to Luonnonvarakeskus. Jokinen Kari and Mäkelä Pirjo have patent #FI128830 granted to Luonnonvarakeskus.

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Appendix A. Supplementary data

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